

SUMMARY OF THE INVENTION:

Calf-chymosin gene is isolated preferably from the fourth stomach of milk fed calf tissues. Recombinant calf-chymosin is produced by cloning chymosin gene with bacterial expression vector pET21b and is transformed to E-coli strain. This E-coli strain containing recombinant calf-chymosin gene is fermented under suitable conditions preferably in a culture medium developed by us. This medium contains the following

Peptone - 12g/l

Yeast Extract - 24g/l

Sodium chloride — 10g/l

Prochymosin produced during fermentation is subjected to denaturation by increasing the pH of the medium to 10-11. The suspension then diluted and the pH reduced to about 8 for effective renaturation of the protein. The prochymosin thus obtained is then acidified for activation and is further processed.

This invention relates to a process for producing recombinant calf-chymosin which comprises the steps of isolating calf-chymosin gene, cloning the same in bacterial expression vector pET21b, transforming said cloned vector into cells of E- coli, fermenting said E-coli strains to produce pro-chymosin, converting said prochymosin to chymosin and subsequently recovering the recombinant calf chymosin. This invention also includes recombinant calf-chymosin having the following aminoacid sequence as setforth in SEQ ID No. 1 and the corresponding gene sequence as setforth in SEQ ID No. 2.

SEQ ID No. 1. Recombinant Calf-Chymosin Protein Sequence

Met A S I T R I P L Y K G K S L R K A L K E H G L L E D F L Q K Q Q Y G I S S K
Y S G F G E V A S V P L T N Y L D S Q Y F G K I Y L G T P P Q E F T V L F D T G
S S D F W V P S I Y C K S N A C K N H Q R F D P R K S S T F Q N L G K P L S I H
Y G T G S M Q G I L G Y D T V T V S N I V D I Q Q T G G L S T Q E P G D V F T Y
A E F D G I L G M A Y P S L A S E Y S I P V F D N M M N R H L V A Q D L F S V
Y M D R N G Q E S M L T L G A I D P S Y Y T G S L H W V P V T V Q Q Y W Q F
T V D S V T I S G V V V A C E G G C Q A I L D T G T S K L V G P S S D I L N I Q
Q A I G A T Q N Q Y D E F D I D C N N L S Y M P T V V F E I N G K M Y P L T P S
A Y T S Q D Q G F C T S G F Q S E N H S Q K W I L W D V F I R E Y Y S V F D R
A N N L V G L A K A I **Stop**

SEQ ID No. 2. Recombinant Calf-Chymosin Gene Sequence

ATG GCT AGC ATC ACT AGG ATC CCT CTG TAC AAA GGC AAG TCT CTG
AGG AAG GCG CTG AAG GAG CAT GGG CTT CTG GAG GAC TTC CTG CAG
AAA CAG CAG TAT GGC ATC AGC AGC AAG TAC TCC GGC TTC GGG GAG
GTG GCC AGC GTG CCC CTG ACC AAC TAC CTG GAT AGT CAG TAC TTT
GGG AAG ATC TAC CTC GGG ACC CCG CCC CAG GAG TTC ACC GTG CTG
TTT GAC ACT GGC TCC TCT GAC TTC TGG GTA CCC TCT ATC TAC TGC AAG
AGC AAT GCC TGC AAA AAC CAC CAG CGC TTC GAC CCG AGA AAG TCG
TCC ACC TTC CAG AAC CTG GGC AAG CCC CTG TCT ATC CAC TAC GGG
ACA GGC AGC ATG CAG GGC ATC CTG GGC TAT GAC ACC GTC ACT GTC
TCC AAC ATT GTG GAC ATC CAG CAG ACA GGA GGC CTG AGC ACC CAG
GAG CCC GGG GAC GTC TTC ACC TAT GCC GAA TTC GAC GGG ATC CTG
GGG ATG GCC TAC CCC TCG CTC GCC TCA GAG TAC TCG ATA CCC GTG
TTT GAC AAC ATG ATG AAC AGG CAC CTG GTG GCC CAA GAC CTG TTC
TCG GTT TAC ATG GAC AGG AAT GGC CAG GAG AGC ATG CTC ACG TTG
GGG GCC ATC GAC CCG TCC TAC TAC ACA GGG TCC CTG CAC TGG GTG
CCC GTG ACA GTG CAG CAG TAC TGG CAG TTC ACT GTG GAC AGT GTC
ACC ATC AGC GGT GTG GTT GTG GCC TGT GAG GGT GGC TGT CAG GCC
ATC CTG GAC ACG GGC ACC TCC AAG CTG GTC GGG CCC AGC AGC GAC

[AMENDED SHEETS]

ATC CTC AAC ATC CAG CAG GCC ATT GGA GCC ACA CAG AAC CAG TAC
GAT GAG TTT GAC ATC GAC TGC AAC AAC CTG AGC TAC ATG CCC ACT
GTG GTC TTT GAG ATC AAT GGC AAA ATG TAC CCA CTG ACC CCC TCC
GCC TAT ACC AGC CAG GAC CAG GGC TTC TGT ACC AGT GGC TTC CAG
AGT GAA AAT CAT TCC CAG AAA TGG ATC CTG TGG GAT GTT TTC ATC
CGA GAG TAT TAC AGC GTC TTT GAC AGG GCC AAC AAC CTC GTG GGG
CTG GCC AAA GCC ATC TGA

In the above sequence, amino acids shown in red indicate sequence variation of chymosin gene of our invention compared to the reported and published sequence.

A recombinant calf-Chymosin protein is set forth in SEQ ID No. 1, wherein the replacement of single amino acid Aspartic Acid (D) with Glycine (G) at position 287 is also covered and is referred to as SEQ ID No.1.

A recombinant calf-Chymosin gene is set forth in SEQ ID No. 2, wherein the replacement of nucleotide GAT with GCC at position 287 is also covered and is referred to as SEQ ID No.2.

PCR amplification of prepro chymosin was performed using the 50ng of 1st strand cDNA with a reverse primer (5'-TGT GGG GAC AGT GAG GTT CTT GGT C-3'), and a forward primer (5'-ATG AGG TGT CTC GTG GTG CTA CTT-3') in a thermal cycler programmed as (step 1 : 95-5'; step 2: 94-30sec; step 3: 54-30sec; step 4: 72-1min; step 5: go to step 2 34 times; step 6: 72-7min; step 7: end). The PCT reaction when analyzed on 1.0% agarose gel showed an amplified band of 1.2kb. The 1.2kb fragment was cut with a sterile blade and the gel slice was dissolved in 500µl of Tris saturated phenol was added and left in liquid nitrogen for a few min. The microcentrifuge tube was allowed to come to room temperature and centrifuged for 5min at 12,000rpm, 4°C. The upper aqueous phase was extracted with phenol : chloroform : isoamyl alcohol (25 : 24 : 1) and DNA was precipitated with 1/10th volume sodium acetate and 2.5 volume ethanol at -70°C for 1 h. DNA was precipitated at 15,000rpm for 15 min. The pellet was dried and dissolved in sterile distilled water. This eluted 1.2kb fragment was ligated at SmaI site of pBSSK+ plasmid, which was then transformed in to TOP10 cells of E.coli. The recombinant clones were selected (blue white screening) and checked with restriction digestion analysis of the plasmids. Recombinant plasmid was taken as a template and a PCR was performed using a forward primer (5'-GAT ATA CAT ATG GCT AGC ATC ACT AGG ATC CCT CTG TAC-3') and reverse primer (5'-GCA GTA AGC TTG ACA GTG AGG TTC TTG GTC AGC G-3') containing Nde I and Hind III sites. An amplified band of 1098bp was observed when the PCR product was analyzed on 1.0% agarose gel. This amplified fragment of 1098bp was eluted from the gel and ligated in pET21b expression vector at Nde I and Hind III sites and transformed in to BL21 cells of E.coli for the expression of the chymosin gene.